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Article (Accepted Version)

Evans, J S, Selden, D, Wu, G, Wright, E, Horton, D L, Fooks, A R and Banyard, A C (2018) Antigenic site changes in the rabies virus glycoprotein dictates functionality and neutralizing capability against divergent lyssaviruses. *Journal of General Virology*, 99. pp. 169-180. ISSN 0022-1317

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Antigenic site changes in the rabies virus glycoproteins dictate functionality and neutralizing capability against divergent lyssaviruses

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Abstract (222 words)

Lyssavirus infection has a near 100% case fatality rate following the onset of clinical disease, and current rabies vaccines confer protection against all reported phylogroup I lyssaviruses. However, there is little or no protection against more divergent lyssaviruses and so investigation into epitopes within the glycoprotein (G) that dictate a neutralising response against divergent lyssaviruses is warranted. Importantly, the facilities required to work with these pathogens, including wildtype and mutated forms of different lyssaviruses are scarcely available and as such this type of study is inherently difficult to perform. The relevance of proposed immunogenic antigenic sites within the lyssavirus glycoprotein was assessed by swapping sites between phylogroup-I and phylogroup-II glycoproteins. Demonstrable intra- but limited inter-phylogroup cross neutralisation was observed. Pseudotype viruses (PTVs) presenting a phylogroup-I glycoprotein containing phylogroup-II antigenic sites (I, II III or IV) were neutralised by antibodies raised against phylogroup-II PTV with the site II (IIb, aa 34-42 and IIa, aa 198-200) swapped PTVs being efficiently neutralised whilst site IV swapped PTV was poorly neutralised. The phylogroup-II glycoprotein specific antibodies raised against PTV containing antigenic site swaps between phylogroup-I and phylogroup-II glycoproteins neutralised phylogroup-I PTVs efficiently, indicating an immunodominance of antigenic site II. Live-lyssaviruses containing antigenic site swapped glycoproteins were generated and indicated that specific residues within the lyssavirus glycoprotein dictate functionality and enable differential neutralising antibody responses to lyssaviruses.

Introduction

The lyssaviruses constitute virus genus of importance to both human and animal health. All viruses within this genus are highly neurotropic and are capable of causing a fatal disease resulting in encephalitis, known as rabies [1]. The lyssavirus genus is classified within the Order Mononegavirales, Family *Rhabdoviridae* and comprises 16 proposed species.

Lyssaviruses have non-segmented (single-stranded) negative sense genomes of between 11-12kbp. The genome encodes 5 proteins: the nucleocapsid (N) protein, the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the large polymerase protein (L). The prototypic lyssavirus is rabies virus (RABV), which is primarily transmitted through the bite of an infected animal and causes a higher burden of disease in both humans and animals than any other lyssavirus. Members of the lyssavirus genus are able to infect and cause disease in a wide host range, with theoretically all mammalian species being susceptible to infection [2]. It is estimated that more than 60,000 people die from rabies annually [1, 3-5]. Despite safe and effective prophylactic and post-exposure tools being available RABV remains endemic across much of Africa and Asia. Fourteen of the 16 proposed lyssaviruses have been associated with the infection of bat species and chiropteran species are generally considered to be the reservoir for the lyssaviruses [6, 7]. Infection of humans with non-RABV lyssaviruses appears to be rare with only 13 human fatalities being documented [8].

Safe and effective RABV vaccines have been available for decades. These vaccines, generally based on inactivated preparations of classical RABV strains, can be used for both pre-exposure (PrEP) and post exposure prophylaxis (PEP). PrEP involves a course of parenteral vaccination according to internationally agreed guidelines that are considered sufficient to achieve an antibody titre of 0.5 IU/ml. This arbitrary value is set as the global cut off for antibody mediated protection against rabies [9]. Whilst highly effective against

RABV, the ability of RABV vaccines to protect against other lyssaviruses is variable or undefined. As such, within the lyssavirus genus, viruses have been classified according to both genetic and antigenic data into phylogroups [10-14]. Phylogroup I includes the classical RABVs alongside Aravan lyssavirus (ARAV), Australian bat lyssavirus (ABLV), Bokeloh bat lyssavirus (BBLV), Duvenhage lyssavirus (DUVV), European bat -1 and -2 lyssaviruses (EBLV-1 and EBLV-2), Gannoruwa bat lyssavirus (GBLV), Irkut lyssavirus (IRKV) and Khujand lyssavirus (KHUV). Phylogroup II encompasses the African Lyssaviruses, namely Lagos bat lyssavirus (LBV-lineages A-D), Mokola lyssavirus (MOKV) and Shimoni bat lyssavirus (SHIBV) whilst phylogroup III has been proposed tentatively due to the divergence of the G and includes West Caucasian bat lyssavirus (WCBV) [13], Ikoma lyssavirus (IKOV) [8, 15] and a further, highly divergent lyssavirus, Lleida Bat Lyssavirus (LLEBV) [16]. The differentiation of viruses into phylogroups is mainly based on the degree of protection afforded by the rabies vaccines against each of the viruses in animal models, although some cross neutralisation studies have also been performed. Limited studies have suggested that there is a low degree of cross neutralisation between phylogroups I and II and also between both phylogroups and WBCV [12]. *In vivo* vaccination-challenge experiments have shown reduced or no efficacy of current licensed rabies vaccines against viruses in phylogroup II (MOKV, LBV, SHIBV) [17] and phylogroup III viruses (WCBV, IKOV) [15, 18, 19]. Evidence also exists for variable vaccine efficacy against phylogroup I lyssaviruses [18, 20-23], which suggests a reduction in vaccine protection that correlates with the antigenic distance of lyssavirus species from vaccine strains. This has most comprehensively been demonstrated using antigenic cartography as a mechanism to define the antigenic relationships between lyssaviruses and both post-vaccinal and lyssavirus-specific sera [12]

The target for neutralising antibody responses against all lyssaviruses is the surface glycoprotein (G). This protein is the major viral component responsible for the induction of a host antibody response and is thought to be the sole target of host neutralising antibodies [24]. Studies with the RABV G protein have defined four major antigenic sites and one minor antigenic site [25-33]. These regions include four continuous epitopes and a discontinuous conformational epitope proposed to be formed through the interaction of two distinct regions (antigenic sites IIa and IIb) within the mature protein. The relative importance of each site for neutralisation of RABV has been previously measured using crude estimates of the number of monoclonal antibodies specific to each site, but not with polyclonal sera. Although important in RABV neutralisation, there is currently no evidence to suggest that the same regions have a similar function within the other lyssaviruses. Still, whilst there is no structural model for the lyssavirus glycoprotein, alignments with the Vesicular Stomatitis Virus pre- and post-fusion glycoprotein structures [34, 35] at least suggest that these antigenic sites are present on the exposed surfaces of the glycoprotein. However, the role of these antigenic sites is undefined, hampering assessment of the threat posed by emerging lyssaviruses, and vaccine design. In this study, the importance of these RABV antigenic epitopes for virus neutralisation across the lyssavirus genus was assessed, using data from both pseudotype viruses and recombinant lyssaviruses generated by reverse genetics. This study demonstrates that individual antigenic sites are highly specific in generating the differential neutralisation pattern seen between the proposed lyssavirus phylogroups against the lyssavirus G protein.

Results

All phylogroup I PTVs are neutralised by sera derived from canine and human vaccinees.

To assess the ability of sera from vaccinated humans and animals to neutralise all viruses within phylogroup I, a panel of PTVs was generated to enable the assessment of a

representative of each lyssavirus species. Two types of RABV specific sera were assessed against this panel: WHO serum, an international standard sera prepared from blood sampled from vaccinated humans that is used as control sera for WHO gold standard serological assays; and Veterinary Laboratories Agency (VLA) serum, a diagnostic control serum obtained from a pool of serum from vaccinated dogs used in serological diagnostic tests performed at APHA, UK. Both sera were used at a dilution of 0.5 IU/ml, the WHO and OIE internationally accepted cut-off titre for seroconversion [9] and a more stringent threshold of 0.1 IU/ml according to dilutions assessed using live virus in the Fluorescent Antibody Virus Neutralisation test (FAVN). The PNA was used to assess the ability of WHO and VLA sera to neutralise each phylogroup I PTV (Figure 1) and all were neutralised by >90% by these sera. Furthermore, all of the phylogroup I PTVs were also completely neutralised by WHO and VLA sera at 0.1 IU/ml. (Supplementary Figure 1).

A humoral response to phylogroup II lyssaviruses neutralises other phylogroup II PTVs

To investigate the degree of cross neutralisation between viruses within phylogroup II, specific sera had to be generated specific for phylogroup II. A PTV expressing LBV lineage B G was selected for inoculation into rabbits to generate sera specific for this G protein. Representative PTVs for all available lyssavirus phylogroups were successfully generated. Dilution of LBV lineage B sera to a neutralising concentration approximately equal to the 0.5IU/ml cut off assigned to seroconversion was performed using a modified version of the FAVN with homologous live LBV lineage B and was expressed as reciprocal titre. This serum demonstrated a strong degree of cross neutralisation within the phylogroup (Figure 2) as had previously been demonstrated for viruses within phylogroup I [22]. Because LBV lineage A, C and D sera were unavailable, investigation into the neutralisation of LBV isolates was limited to LBV lineage B specific serum. The LBV B serum was able to

neutralise the three LBV PTVs tested as well as a representative MOK and SHIBV PTV to >90%. LBV serum demonstrated a lower degree of neutralisation of WCBV and IKOV PTVs with values of 27% and 60%, respectively.

Alterations to antigenic domains within the lyssavirus G affect both functionality and neutralisation profiles of the mutated proteins.

To further investigate the relative importance the five defined antigenic epitopes, a series of recombinant PTVs were developed that had the defined antigenic epitopes swapped between a phylogroup I virus (CVS) and a phylogroup II virus (LBV lineage B). Both individual antigenic site mutants [36] were utilised alongside G proteins that had all five antigenic epitopes exchanged between the two viruses (Figure 3). Once plasmids had been constructed, the ability to generate PTs with each of the single site swaps (SSS) was assessed alongside the ability to generate the full site swap (FSS) mutants. Initially, functionality of mutated forms of the G protein were assessed through their relative ability to generate titres as PTVs. Then, where titres were achieved, the ability of standard sera to neutralise these recombinant PTVs were assayed. All attempts to generate a PTV containing the CVS G with all five antigenic sites being swapped with those of LBV (CVSFSS) failed to generate titres of 200 TCID₅₀/ml and so could not be used in the PNA.

The WHO, VLA and LBV lineage B sera described earlier were next used to assess the antigenic effect of the site swaps. All CVS based PTVs including the CVS wildtype PTV were almost completely neutralised by both serum samples specific for RABV. The CVS wildtype PTV was not neutralised by the LBV B serum, however a number of single and multiple antigenic site swap mutants were neutralised by the LBV B specific sera, with those mutants containing the LBV site II epitope(s) being most strongly affected (Supplementary

Figure 2). CVS to LBV IIb was neutralised by 98% whilst CVS to LBV IIaIIb and CVS to LBV IIa were neutralised by 80% and 65%, respectively. Alterations to sites I and III also affected the neutralisation profiles observed although to a lesser extent. The exchange of site IV had little effect on the neutralisation profiles observed.

The reciprocal assay was performed with the LBV to CVS antigenic site swaps, again utilising the same panel of sera described previously (Supplementary Figure 3). The LBV B wildtype PTV was neutralised by the LBV B specific sera although neutralisation of the LBV B wildtype PTV was also observed with the RABV specific sera. Furthermore, the LBVFSS PTV containing all of the antigenic epitopes of CVS was neutralised by both the RABV and LBV specific sera. Each of the single site swap PTVs were neutralised by the LBV specific sera although some of the mutants, LBV to CVS sites I, IIb, IIaIIb and III, were neutralised to a greater degree by the RABV sera than the LBV sera suggesting that these epitopes may play more prominent roles in neutralisation.

Sera generated against mutated PTVs demonstrated differential neutralisation of wildtype PTVs

To further investigate the role of antigenic epitopes in the G protein, a series of mutated and wildtype PTVs were inoculated into rabbits to generate sera specific for each mutant. Four mutant PTVs were selected, CVS to LBV I, LBV to CVS I, LBV to CVS IIaIIb and LBVFSS, on the basis of earlier results. The CVS to LBV I, LBV to CVS I, LBV to CVS IIaIIb and LBVFSS specific sera were run against wildtype CVS and LBV PTVs at 0.5 IU/ml (Figure 4). The CVS to LBV I serum neutralised the CVS PTV but also neutralised the LBV PTV by 47%. In the reciprocal experiment the LBV to CVS I serum completely neutralised the LBV PTV but only neutralised the CVS PTV by 7%. Interestingly, the LBV to CVS

IlaIIb serum neutralised the CVS PTV more strongly than the LBV PTV; by 93% and 64%, respectively. Finally, the LBVFSS specific serum neutralised both CVS and LBV PTVs to a similar extent; 51% and 56% respectively.

Mutations to antigenic sites within the wildtype glycoproteins affect virus rescue

The observation that the CVSFSS PTV could not be generated, even following back mutation of sites IIa and IIb, led to speculation that the mutations incorporated into the glycoprotein had rendered it non-functional. Interestingly, the LBVFSS PTV could be generated and sufficient PTV titre reached to perform the PNA. To further investigate glycoprotein functionality following mutation a panel of full length clones was generated using a SAD B19 vaccine strain plasmid backbone [37, 38] with the wildtype glycoprotein gene being replaced with the mutated versions described for the PTVs. Despite repeated rescue attempts varying plasmid concentration, transfection reagent:DNA ratios and cell confluency the cSN-CVSFSS plasmid failed to generate live virus that could be passaged *in vitro*. In contrast the cSN, cSN-CVS, and cSN-LBVFSS constructs produced viable virus and the expression of N was assessed by direct immunofluorescence (Data not shown). These three viruses were passed until confluency was achieved. Whilst the cSN and cSN-CVS virus reached 100% confluency after only 5 passages, the cSN-LBVFSS virus appeared restricted in growth and was unable to generate extensive foci of infection even after 10 passages.

A multistep growth curve with rescued viruses demonstrated a reduction in titre in the recombinant viruses compared with cSN; cSN reached a peak titre of 8.35×10^8 ffu/ml at 72 hours post infection (hpi) whilst cSN-CVS grew maximally to 5.84×10^3 ffu/ml (Figure 5). cSN-LBVFSS was detected from 24 hpi at a very low level of 4.85×10^1 ffu/ml but could not be detected at 96 hpi.

Assessment of vaccine protection and pathogenicity of recombinant viruses in the murine model

Considering the effect of antigenic site swaps on fitness *in vitro*, it was important and relevant to vaccine design to assess their pathogenesis *in vivo*. Both peripheral pathogenesis experiments and vaccination challenge experiments were undertaken. To assess peripheral pathogenicity in unvaccinated mice, groups of naïve mice were inoculated peripherally via the intramuscular route. Previous studies have demonstrated that to demonstrate pathogenicity in this mouse breed following intramuscular peripheral inoculation at least 1000ffu/50µl is required (unpublished data). Therefore, cSN and cSN-CVS were both inoculated at 1000ffu/50µl. However, despite repeated passages and attempts to concentrate the virus, the titre of cSN-LBVFSS could not be increased above 200ffu/50µl and so this dose was inoculated peripherally. To control for cSN-LBVFSS, a group of mice were peripherally inoculated with 1000ffu/50µl of a wildtype LBV lineage B. All peripherally inoculated mice survived infection with the exception of those inoculated with cSN-CVS where 40% succumbed on day 8 post infection and the wildtype LBV isolate where 20% developed clinical disease at 8 days post infection (Figure 6a). Of those that survived infection, 60% of the cSN-LBVFSS peripherally infected mice generated a serological response of >0.5IU/ml. All unvaccinated mice infected with LBV seroconverted strongly against LBV and 50% (n=2/4) had a low neutralising titre against CVS (Figure 7).

For assessment of vaccine protection, four groups of mice (n=10/group) were vaccinated and challenged on day 28 post vaccination. Four groups of 5 mice were mock vaccinated with phosphate buffered saline (PBS) using the same vaccination schedule. All vaccinated mice seroconverted with reciprocal titres equal to or greater than the WHO 0.5IU/ml control serum

by FAVN (data not shown) and a pool of sera from mock vaccinated mice had a titre that indicated serological naïvety in unvaccinated animals.

Unvaccinated mice inoculated with either 100ffu/30µl of cSN or cSN-CVS via the IC route developed clinical disease consistent with lyssavirus infection at 7 days post infection (Figure 6b). Unvaccinated mice inoculated with 100ffu/30µl of cSN-LBVFSS did not develop clinical disease throughout the duration of the experiment. All unvaccinated mice inoculated IC with 100ffu/30µl of wildtype LBV, succumbed by day 7 post infection.

All vaccinated mice survived IC inoculation with cSN and cSN-CVS. Of the vaccinated mice that were challenged with LBV, 80% succumbed to infection between days 7 and 9 (Figure 6c). The two remaining LBV vaccinated mice survived up the end of the experiment and strongly seroconverted against both CVS and LBV with high virus neutralisation reciprocal titres against both LBV and CVS (data not shown). No mice challenged IC with 100ffu/30µl of cSN-LBVFSS developed clinical disease. However, from the cSN-LBVFSS challenged mice, all mock vaccinated, vaccinated and peripherally challenged unvaccinated mice seroconverted with very high serological responses to CVS (Figure 7). In contrast none of these mice generated serological responses capable of neutralising LBV (data not shown).

Quantifying the antigenic effect of known antigenic sites

The antigenic relationships of CVS, LBV and LBVFSS recombinant viruses are illustrated in the antigenic map (Figure 8). The positions of CVS and LBV correspond to those determined previously, with CVS close to but distinct from other RABVs and LBV antigenically distant from all other lyssavirus species [12]. Previous studies have demonstrated that substitution of the complete glycoproteins between different lyssaviruses causes a complete shift in

antigenic phenotype to that of the G protein [38]. The antigenic mapping performed here has demonstrated that although swapping all previously defined major antigenic sites (LBVFSS) in G has had a significant effect on the antigenic phenotype of the resulting virus, it has not caused a complete phenotypic switch as seen with a replacement of the wildtype G. LBV with antigenic sites of CVS (LBVFSS) is positioned equidistant to the two wildtype viruses on which it is based (CVS and LBV). Relationships of sera raised against PTVs were also investigated using the same cartographical techniques (Figure 8). Serum raised against PTVs with only site I altered are close to the original or wildtype virus, whereas sera raised using a PTVs with site IIaIIb, and that with all sites substituted (FSS), are closer to the LBVFSS virus than to either CVS or LBV. Although it is not possible to account for individual variation in serological response with these single sera, these results suggest that site IIaIIb has a larger effect on the antigenicity than antigenic site I in this system.

Discussion

The absence of a crystal structure for the lyssavirus G precludes a thorough antigenic assessment of the lyssavirus G protein. Modelling the antigenic epitopes of lyssaviruses onto existing rhabdovirus G structures at least demonstrates that antigenic sites defined by monoclonal antibody typing for rabies are potentially located in exposed positions on the ectodomain of G [39] but this does not necessarily reflect the position or role of these regions in the mature native protein. As such the antigenic repertoire that precludes vaccine derived neutralisation for the lyssaviruses remains difficult to define.

Data generated here using the PTV assay confirmed that cross neutralisation occurs within phylogroup I using standardised sera at both 0.5 IU and 0.1U/ml. This corroborates existing

293 studies and suggests cross neutralisation within phylogroup I, both to a protective titre of
294 anti-RABV serum but also to an apparently sub-protective but clearly neutralising dilution of
295 serum. Previous studies have suggested that antibody titres lower than the internationally
296 recognised cut-off (0.5IU/ml) are protective although test sensitivity makes a precise
297 definition of this cut-off difficult [9, 40]. Regardless, varying levels of cross neutralisation
298 within phylogroup I have been demonstrated previously whereby vaccination with the
299 Human Diploid Culture Vaccine (HDCV) was able to protect more efficiently against CVS
300 and EBLV-2 infection than ABLV or EBLV-1[21, 22]. Antigenic cartography has further
301 enabled assessment of antigenicity and studies have suggested that ABLV and KHUV may
302 be antigenically identical to the RABV isolates assessed. IRKV, ARAV and EBLV-1 are also
303 believed to be closely related antigenically to RABV with EBLV-2 being more divergent
304 amongst the isolates assessed [12]. Several studies have suggested a need for higher
305 neutralising antibody titres to effectively neutralise non rabies phylogroup I lyssaviruses [22].
306 A single report of rabies vaccination protecting a dog from a phylogroup II virus exists
307 whereby an animal vaccine, Rabiffa, appeared to protect dogs against challenge with LBV
308 [20]. Observations of antigenic variation among phylogroup I viruses using live viruses, that
309 contrast to studies with PTVs, may indicate disparity between the level of surface antigen
310 presented on a PTV compared to a live virus. A PTV represents a pleiomorphic sack with
311 unregulated amounts of G presented on its envelope in an uncontrolled manner. In contrast,
312 the rhabdovirus virion represents a highly ordered bullet shaped virion with peplomers of
313 trimeric G studded tightly across its surface. As such it is clear that the neutralisation of one
314 is likely more readily achieved than the other. Certainly, this increased sensitivity of the PNA
315 is something that has been observed before with influenza and lyssavirus PTVs [41, 42] and
316 is something that should be considered when assessing neutralisation, especially where
317 neutralisation reflects protection from the development of a fatal infection. Neutralisation

profiles generated for neutralisation of PTVs using novel monoclonal antibodies have reiterated this [36]. Still, as previous studies have demonstrated that antibody responses to endogenous proteins within PTVs do not influence neutralisation of PTVs they can generally be considered a useful surrogate for neutralisation tests that avoid the use of live pathogen and have utility as vaccine candidates, although non-integrating PTV systems may be required to avoid potential safety concerns [43].

Although inter-phylogroup cross-neutralisation is not expected for the lyssaviruses, intra-phylogroup neutralisation has not been assessed for all officially recognised lyssaviruses [17]. Sera from a group of *Eidolon helvum* fruit bats, a reservoir host for Lagos Bat Virus, appeared to be specific for lineage 'A' LBV with no neutralising activity against lineage B LBV [44]. In contrast, assessment of phylogroup II sera with a range of PTVs based on phylogroup II glycoproteins indicated a high degree of cross neutralisation within phylogroup II. The lyssavirus cross-reactivity conundrum is exacerbated by data from wild caught bats where sera have been suggested to be highly specific for individual lyssaviruses. Sera from 76 insectivorous bats that reacted against WCBV were unable to neutralise other lyssaviruses, including LBV, MOKV, RABV and DUVV. Interestingly, a serum sample from a single bat also strongly neutralised DUVV [13]. Partial cross neutralisation of WCBV and IKOV by the LBV lineage B specific serum was observed in this study suggesting some cross neutralisation even where 54% (WCBV) and 48% (IKOV) amino acid identity is seen. Previous investigations indicated that antibodies induced by rabies vaccination provide no protection against infection with phylogroup II or III viruses, even at concentrations 20 times greater than those which neutralise phylogroup I constructs [18, 19]. This likely reflects that whilst PTVs have shown great utility in diverse applications, the amount of G presented on pleomorphic PTV particles in relation to that on naturally occurring virions requires further

investigation and cross-neutralisation afforded by sera to live replication competent viruses is required where isolates are available.

The relative lack of inter-phylogroup neutralisation led to studies to investigate individual residues within the glycoprotein and their role in immunodominance. Understanding immunologically important domains has implications for assessing risk posed by emerging lyssaviruses, and for vaccine design. Historically, several immunodominant epitopes have been described for the rabies G protein and the role of these epitopes investigated. From the PNAs utilising PTVs containing Gs with individual site swaps, three antigenic sites were highlighted as being potentially important for both a phylogroup I and II neutralisation responses. The CVS to LBV site swap assay showed that antigenic site II mutants were most strongly neutralised by both RABV and LBV specific sera indicating the role site II in generating antibody response against LBV. CVS PTVs containing LBV antigenic site II demonstrated the potential importance of this discontinuous conformational epitope.

The reciprocal assay assessing LBV to CVS site swaps yielded a different pattern of neutralisation. The neutralisation of the wildtype LBV PTV, by both the VLA and WHO serum, was unexpected and may again reflect a lower density of G on the surface of PTV particles. This requires further investigation although rare instances of rabies vaccine protecting animals against LBV challenge have been described [20]. Interestingly, data from the individual site swap assay suggested that for the LBV to CVS site swaps, exchange of sites I and III did not affect neutralisation by any of the sera tested. There is little evidence in the literature defining the importance of individual antigenic sites in lyssavirus neutralisation yet this study suggest that the generation of a neutralising response that neutralises phylogroup I lyssaviruses may rely on antibodies directed against antigenic sites I (aa 226-

231) and III (aa 330-338). In contrast the data suggests that neutralisation of phylogroup II viruses is more dependent on neutralising antibodies directed against antigenic site II (IIb, aa 34-42; IIa, aa 198-200). Previous studies have suggested that sites II and III are the most important for a CVS neutralising response in *H-2^d* mice [26]. The importance of sites I, II and III is also supported by the finding that a large number of neutralising anti-glycoprotein mAbs are specifically directed towards them [25].

Antigenic cartography with polyclonal sera indicated that there are regions of the glycoprotein, outside of the established antigenic sites that may significantly affect antigenicity. The cSN-LBVFSS recombinant virus was positioned equidistant between the wildtype LBV and the wildtype RABVs on the antigenic map suggesting that the introduced mutations had affected the overall structure of the G protein and its ability to neutralise different lyssaviruses. This novel observation shows that the alteration of specific antigenic sites can be used to measure antigenic effects. Investigation into the neutralising capabilities of rabbit sera raised using site-directed-mutant viruses, suggested that the antigenic site swaps had altered the neutralisation. The CVS to LBV I serum versus the CVS PTV and the LBV PTV showed a very similar neutralisation profile to the reciprocal assay using the antigenic site swapped PTV whereas the LBV to CVS site I results were less similar, indicating LBV site I is more immunogenic than CVS site I. The CVS to LBV PTV panel (Supplementary Figure 2) suggested that sites I, III and IV were less important for neutralisation by serum specific to LBV B. LBV to CVS I serum did not neutralise CVS PTV whereas the LBV B to CVS IIaIIb serum did (Figure 4). This suggests that site II is more important than site I in stimulating a specific antibody response. The LBVFSS serum was able to neutralise both CVS PTV and LBV PTV by approximately 50% (Figure 4) which was in contrast to the ability of both RABV and LBV specific sera to strongly neutralise

LBVFSS PTV (Supplementary Figure 3). The differences between these neutralisation profiles may be due to individual differences in response to the antigens driven by innate differences between rabbit and mouse immune responses as the antigenic sites were originally mapped using murine antibodies [25]. Results may also be skewed through differences in the presentation of G in PTVs and further assessment with live viruses is warranted.

Where functionality was assessed using recombinant viruses, the insertion of heterologous glycoproteins into the cSN backbone had affected the ability of the viruses to grow. The complete failure of cSN-CVSFSS to rescue or grow to a detectable level mimicked attempts to produce the CVSFSS PTV. Both of these findings suggested that the mutations made to the antigenic sites had affected the functionality of this glycoprotein. Antigenic cartography corroborated the effect of antigenic site mutation with the positioning of sera and viruses on the map (Figure 8). The titres recorded correlated with the growth kinetics data (Figure 5) as cSN-LBVFSS had the slowest growth and the lowest peak titre likely mediated by the presence of heterologous M and G proteins present. The precise nature of interaction between the M and G remains undefined although for other viruses, interactions between homologous M and G are important [45-47]. For rabies G and M interactions drive optimal budding of nascent viral particles [48] with the cytoplasmic domain of the G protein playing the key role in this interaction [45, 49]. The detrimental effect of replacing cSN G with a heterologous lyssavirus G was similar to findings from a previous study which incorporated the G proteins from EBLV-1 and EBLV-2 into cSN [38]. The molecular basis for this finding remains to be investigated and the effect of residue mutation on glycoprotein functionality requires further assessment.

Vaccination challenge experimentation demonstrated that almost all mice that were mock vaccinated and challenged IC with recombinant viruses succumbed to infection and were humanely terminated by day 7 post infection (Figure 6a). The exception to this was the group of mice that received cSN-LBVFSS that survived the duration of the experiment. The seroconversion of the mock vaccinated cSN-LBVFSS challenged mice, the anamnestic response to cSN-LBVFSS challenge in vaccinated mice and the seroconversion of 60% of the unvaccinated, peripherally challenged mice suggests that despite poor in vitro growth, this virus was able to replicate in vivo but was likely cleared in the absence of a productive infection (Figure 7). Whilst all of these survivors seroconverted strongly against CVS, no serum neutralising titre was detected against LBV which may indicate a true dominance of the antigenic site(s) of CVS located within the chimeric LBVFSS glycoprotein. Conversely, 50% of the mice peripherally infected with LBV seroconverted against both LBV and CVS whilst the remainder seroconverted against LBV but serum had no neutralising activity against CVS. This suggests the potential for variation in neutralising responses generated between animals. Whilst rarely reported, seroconversion in the absence of clinical disease has been demonstrated for lyssaviruses although mechanisms of viral clearance remain to be described [50].

The present study has indicated that: individual antigenic sites within the lyssavirus G can affect neutralisation profiles; pseudotype particles may be utilised as potential vaccine candidates but have limitations when assessing neutralising profiles; and importantly that immunodominant epitopes identified through monoclonal antibody mapping can differ when comparing responses to whole antigen in polyclonal sera. This latter point is of potential significance as the determination of immunodominant epitopes through monoclonal antibody binding is an artificial process in comparison to protein structure recognition by polyclonal

sera. Certainly, it is likely that other, undefined immunologically important epitopes outside of those assessed here influence the repertoire of neutralising antibodies produced following vaccination. Regardless, the continued discovery of lyssaviruses that are genetically closely related to RABV [51] as well as those that are highly divergent [16] drives the need for further investigation into factors that contribute to a protective immune response across the genus. In conclusion, whilst rabies constitutes the biggest threat to human and animal health, the threat of lyssaviruses to human populations remains undefined and as such further studies that determine factors associated with cross-phylogroup neutralisation are warranted.

Materials and Methods

Cells Baby hamster kidney cells (BHK-21) and Human embryonic kidney cells (HEK 293T/17) were grown and maintained as described previously [52]. All cell lines were maintained at 37°C in a 5% CO₂ incubator (Binder).

PTV plasmid construction, transfection, titration and neutralisation assays (PNA)

Human immunodeficiency virus (HIV) based PTVs were generated using plasmid pI.18 that was prepared to accept insertion of lyssavirus glycoproteins following digestion by restriction enzymes [52]. Transfections were carried out with Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's instructions and as described previously [52]. The titres of harvested PTVs were determined by 50% tissue culture infective dose (TCID₅₀) [52]. A negative cut off value was defined as the average cells alone luminescence multiplied by 2.5 [52]. The last dilution at which all wells for each PTV were positive was used to determine the PTV titre in TCID₅₀/ml calculated using the Spearman-Kärber method [53]. Reporter gene activity was measured on a Glomax 96 microplate luminometer (Promega) and for the percentage neutralisation test, the degree of neutralisation was calculated as a reduction in the

level of luminescence compared to virus not exposed to serum. Each assay was carried out in triplicate and a mean average percentage neutralisation was calculated. For the serum titre test the 50% end point dilution (where complete neutralisation stopped in 50% of the wells) was also calculated using the Spearman-Kärber method [53]. Back titration of PTV was carried out alongside each PNA using the same method as that for initial titration to ensure consistent input of PTV.

Full length plasmid construction Glycoproteins of interest were cloned into the cSN backbone, a reverse genetics system based on the SAD B19 of rabies as described previously [38]. Constructs were generated including those containing swaps between phylogroups of all five antigenic epitopes, termed Full Site Swaps (FSS) (Figure 3). The glycoprotein sequences of the rabies Challenge Virus Standard (CVS), CVSFSS and LBVFSS were each cloned into a vaccine strain backbone plasmid, cSN in place of its wildtype glycoprotein sequence. Primers to amplify different G open reading frames (ORFs) were generated to incorporate *HpaI* and *NheI* (Promega) restriction enzyme sites for downstream cloning into cSN. Following ligation and transformation, clones were checked by restriction enzyme digestion and plasmid sequencing.

Virus rescue, titration and growth curves Virus rescue was performed as described previously [38] using fowlpox T7 (FPT7) to provide sufficient T7 RNA polymerase. After one hour of incubation at 37°C the FPT7 was removed, the cells washed with PBS and each well was transfected with 1µg of pN, 1µg of pP, 1µg of pL and 2µg of genome plasmid using FuGENE6. Plates were incubated for 48-72 hours prior to harvest. Rescued virus was passaged until 100% infectivity was reached and titrated as previously described [54]. Virus titres were determined as focus forming units per ml (ffu/ml). Multistep growth curves were

conducted as previously described [38]. At each required time point, 100µl of supernatant was harvested, frozen at -80°C then thawed and titrated in triplicate on BHK cells.

Ethics statement

All studies involving animals were carried out within the appropriate high containment facilities at the Animal and Plant Health Agency (APHA), UK. All mouse and rabbit studies were conducted under HO project licenses PPL70/7394 and PPL 70/8275, respectively, following internal ethical review at APHA. All animals utilised in the study were given access to food and water *ad libitum*.

Generation of PTV specific sera in rabbits A panel of PTVs containing wildtype or mutated lyssavirus G proteins was generated and inactivated by the addition of β-propiolactone (1/4000 final dilution) and incubation at 4°C for 19 hours followed by 1 hour at 37°C. Aliquots of inactivated PTV were stored at -20°C until required. Each PTV was mixed with Montanide™ ISA 50 V2 (Seppic) at a 1:1 ratio prior to inoculation. One rabbit was inoculated per PTV. Prior to administration, inoculation sites were shaved and Emla cream (AstraZeneca) was applied. Each rabbit was inoculated at four sites subcutaneously followed by two sites intramuscularly on days 0, 21, 28 and 35. On day 43 all rabbits were anaesthetised and blood was removed via cardiac puncture under terminal anaesthesia. Following collection, blood was subjected to centrifugation at 2500 rpm (860 x g) for 10 minutes, and cleared serum was harvested. Sera were heat inactivated at 56°C for 30 minutes then divided across 500µl aliquots and stored at -20°C until required.

Murine studies

517 **Mice** Three to four week old female CD1 mice (Charles River) were microchipped using
518 Trovan chips to enable identification and housed as described above.

519

520 **Vaccination of mice** The human rabies vaccine VeroRAB (Novartis) was reconstituted as
521 per the manufacturer's instructions in 1ml of sterile water immediately prior to vaccination of
522 the mice. The reconstituted vaccine was diluted 1 in 20 in sterile filtered deionised water.
523 Vaccine was introduced via intraperitoneal injection of 500µl to the lower right hand
524 quadrant of the abdomen on days 0 and 14.

525

526 **Blood sampling by tail bleed** At 21 days post vaccination the dorsal tail vein of each mouse
527 was nicked under anaesthesia using a scalpel blade and blood was collected in CB 300 tubes
528 (Sarstedt). Following collection, blood samples were stored at 4°C overnight prior to
529 centrifugation at 2500 rpm (860 x g) for 10 minutes after which the serum was separated
530 from the blood pellet. These serum samples were heat inactivated at 56°C for 30 minutes and
531 stored at -20°C until required.

532

533 **Intracranial challenge with virus** Mice were challenged intracranially with 100ffu/30µl of
534 infectious recombinant virus. Following inoculation with live virus mice, were checked twice
535 daily. Clinical scores were recorded for each mouse according to an established clinical score
536 system with defined humane end points [55]. Any mice showing signs of clinical disease of
537 score 3 were anaesthetised, blood collected by cardiac puncture and euthanised by cervical
538 dislocation.

539

540 **Peripheral infection with virus** Mice were inoculated into the footpad with 1000ffu/50µl of
541 each virus under anaesthesia. Once anaesthetised, mice were placed into a holding container

with a small hole, through which the left hind limb was extended to enable inoculation. Mice were monitored for 28 days post infection. Clinical signs were scored and mice terminated as described [55].

Antigenic cartography Using techniques described previously [12, 56] an antigenic map was constructed illustrating the relationships of CVS, LBV and LBVFSS according to the titres of reference sera. Briefly; a target distance from a serum to each virus was derived by calculating the difference between the logarithm (\log_2) reciprocal neutralisation titre for that particular virus and the \log_2 reciprocal maximum titre achieved by that serum (against any virus). Antigenic cartography [56] implemented using ACMACS (www.antigenic-cartography.org) was then used to optimise the positions of the viruses and sera relative to each other on a map, minimising the sum-squared error between map distance and target distance. 100 random restart optimisations created multiple maps which were ranked in order of total error and quantitatively compared to each other, and previous maps with the same viruses, for self-consistency. A total of 41 sera (37 control sera and four new sera generated in this study) were used to position the viruses on the map.

Acknowledgements

This study was funded by DEFRA grant SE0426 and SE0431. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. JSE was supported in a PhD studentship through this grant. We thank the animal care staff at APHA for assistance with experimentation and the APHA Animal Welfare ethics Review Board for ethical appraisal of studies.

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Figure legends

Figure 1 Neutralisation profile of phylogroup I PTVs against 0.5 IU/ml RABV specific

sera. WHO serum from vaccinated human and VLA serum from vaccinated dogs were diluted to 0.5 IU/ml. Error bars represent standard deviation about the mean of three replicate in-assay neutralisation tests.

Figure 2 Cross neutralisation within a phylogroup II lyssavirus PTV panel. PTV

neutralisation assay. PTVs representing all members of phylogroup II plus phylogroup III WCBV and the highly divergent IKOV were run against an LBV lineage B PTV specific rabbit derived polyclonal serum diluted to 0.5 IU/ml. Error bars represent standard deviation about the mean of three replicate in-assay neutralisation tests.

Figure 3 Schematic of the lyssavirus glycoprotein demonstrating the positions of defined

antigenic sites. Glycoprotein monomers are shown with antigenic sites labelled. Examples given are: CVSFSS whereby genetic portions from CVS are shaded grey and the antigenic sites of LBV are white; The reciprocal, LBVFSS is shown with regions corresponding to CVS being white and the antigenic sites of LBV being shaded grey; and LBV-CVS IIaIIb whereby just CVS sites IIa and IIb are swapped into the LBV G. Defined antigenic sites are numbered following removal of the signal peptide. TM: transmembrane; NH₂: amino terminus; COOH: Carboxyl-terminus. (Adapted from 39)

Figure 4 Assessment of neutralisation profiles for sera derived from rabbits inoculated

with antigenic mutant PTVs. The four sera directed against antigenic site swap mutants were diluted to 0.5 IU/ml and run against wildtype CVS and LBV PTVs. A 2-way ANOVA was used to determine any significant differences between neutralisation of each PTV by the

test sera compared to the lack of neutralisation by negative dog serum (0% neutralisation by negative dog serum not shown). Significant differences are indicated by asterisks; * $p \leq 0.05$. Error bars represent standard deviation about the mean of three replicate in-assay neutralisation tests.

Figure 5 Growth kinetics of recombinant lyssaviruses *in vitro*. Multiple step growth curves of each virus starting from MOI 0.01.

Figure 6 *In vivo* assessment of recombinant viruses. (A) Survival curve of mice challenged peripherally. Each mouse was challenged with 1000ffu/50 μ l of virus via the foot pad, except the low titre cSN-LBVFSS which was inoculated neat at 200ffu/50 μ l. $n = 5$ per virus. (B) Survivorship of mock vaccinated (PBS) mice ($n = 5$ /virus). Mice were infected with 100ffu/30 μ l of virus via the intra cranial route. (C) Vaccinated mice were challenged with 100 ffu/30 μ l of virus via the IC route on day 28 post vaccination. Survivorship from day 28 for 21 days is shown ($n = 10$ per virus).

Figure 7 Serological responses in mice inoculated with cSN-LBVFSS. Mock vaccinated (PBS) mice, vaccinated mice and unvaccinated peripherally challenged mice were assessed for serological responses to CVS. Neutralising antibody titre values are in IU/ml but plotted on a logarithmic scale for clarity. The defined 0.5IU/ml protective neutralising antibody titre is shown as a dotted line and is labelled on the y axis.

Figure 8 Antigenic cartography demonstrating relative positioning of antigenic site mutant derived sera and viruses on a three dimensional map. Different coloured spheres represent viruses as labelled. The mutations incorporated into cSN-LBVFSS have altered its

748 antigenicity, positioning it closer to CVS. Sera raised against single site mutants have also
749 been displaced (labelled grey boxes).

750

751

Supplementary Figure 1 Neutralisation profile of phylogroup I PTVs against 0.1 IU/ml

RABV specific sera. PTV neutralisation assay. WHO serum from vaccinated human and VLA serum from vaccinated dogs were diluted to 0.1 IU/ml. Error bars represent standard deviation about the mean of three replicate in-assay neutralisation tests.

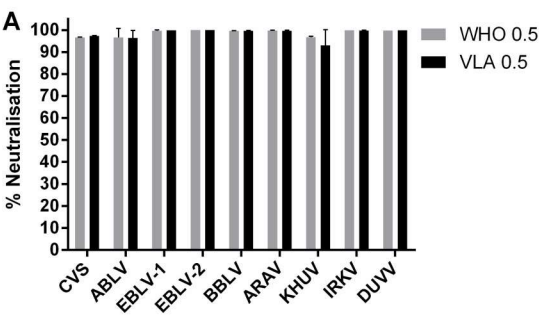
Supplementary Figure 2 CVS antigenic site swap PTV neutralisation profiles. The result

of the CVS panel of antigenic site swap mutants PNA against both phylogroup I specific sera, WHO and VLA as well as phylogroup II specific sera, LBV. All sera were diluted to 0.5 IU/ml. A two way ANOVA was used to determine significant differences between neutralisation of each PTV and neutralisation of CVS by its homologous WHO and VLA sera. Significance is indicated by an asterisk; * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0001$. Error bars represent standard deviation about the mean of three replicate in-assay neutralisation tests.

Supplementary Figure 3 LBV antigenic site swap PTV neutralisation profiles. The result

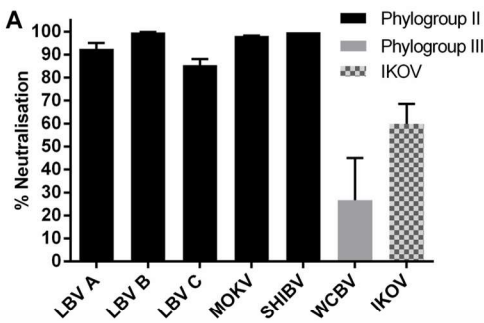
of the LBV panel of antigenic site swap mutants PNA against both phylogroup I specific sera, WHO and VLA as well as phylogroup II specific sera, LBV. All sera were diluted to 0.5 IU/ml. Error bars represent standard deviation about the mean of three replicate in-assay neutralisation tests.

Evans et al Figure 1



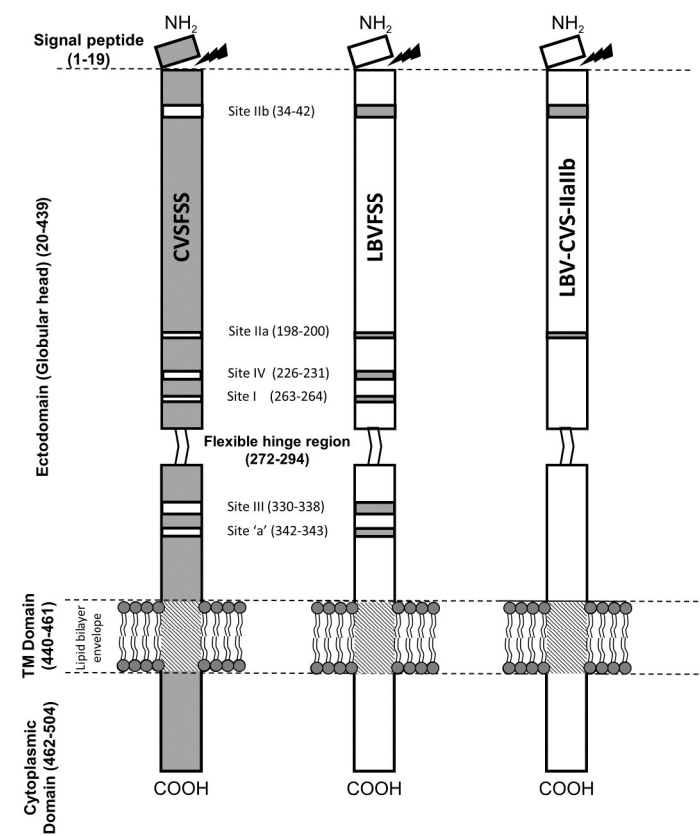
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Evans et al Figure 2

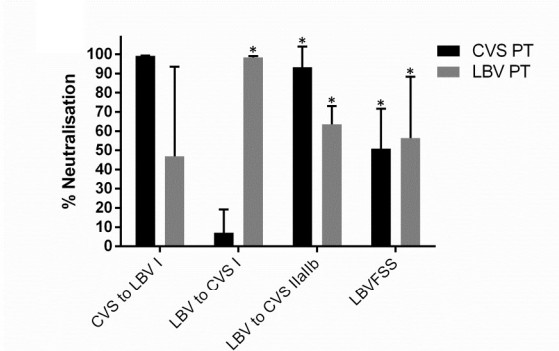


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Evans et al Figure 3

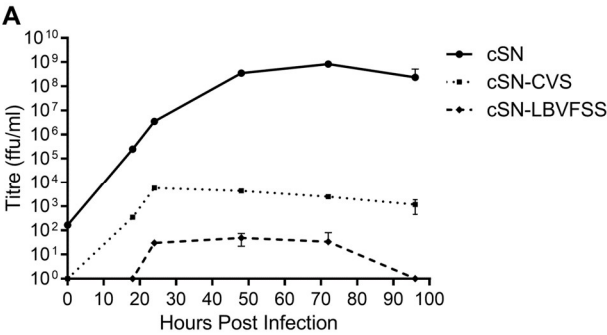


Evans et al Figure 4



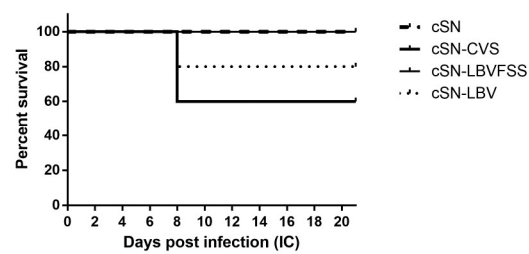
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Evans et al Figure 5



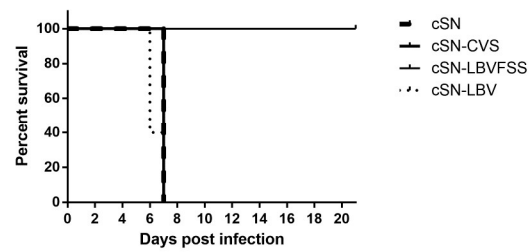
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a- Peripheral infection



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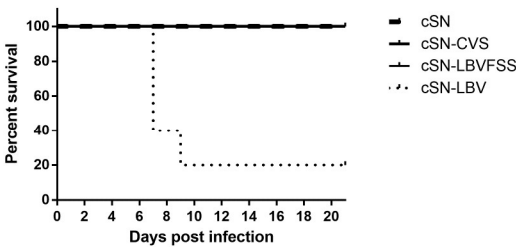
b- Mock vaccinated then challenged (IC)



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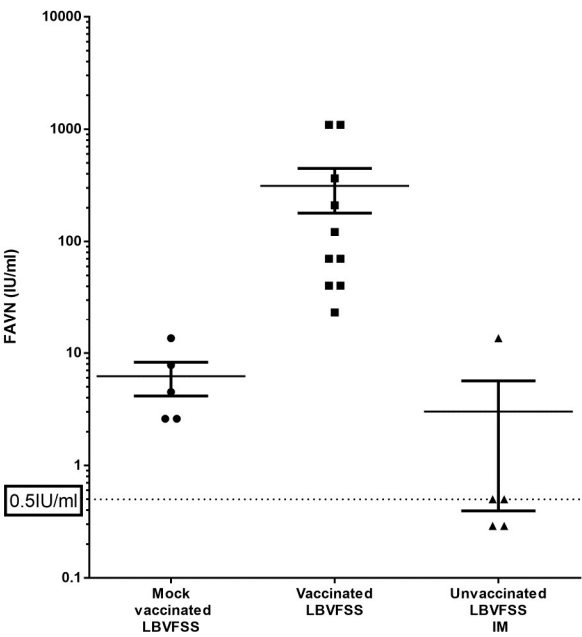
Evans et al Figure 6

c- Vaccinated then challenged (IC)



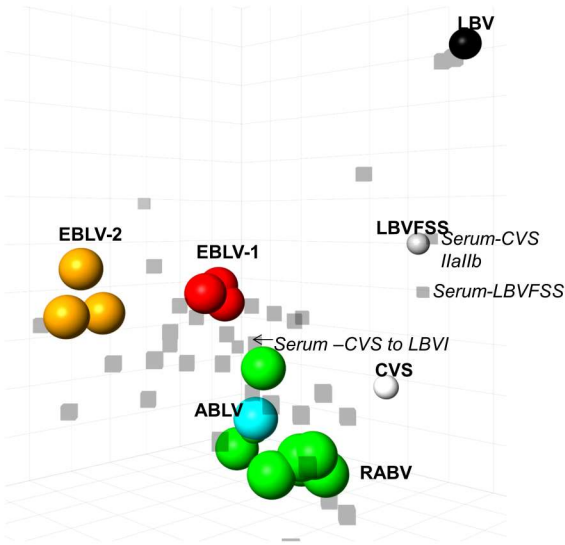
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Evans et al Figure 7

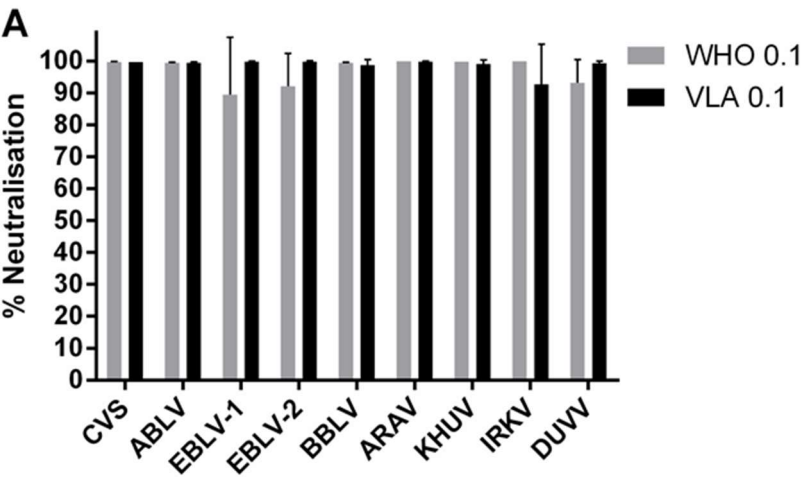


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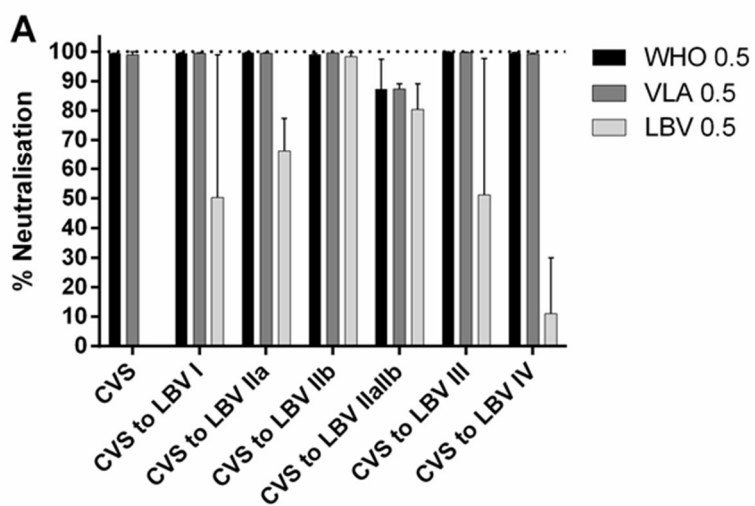
Evans et al Figure 8



Evans et al Supp. Figure 1

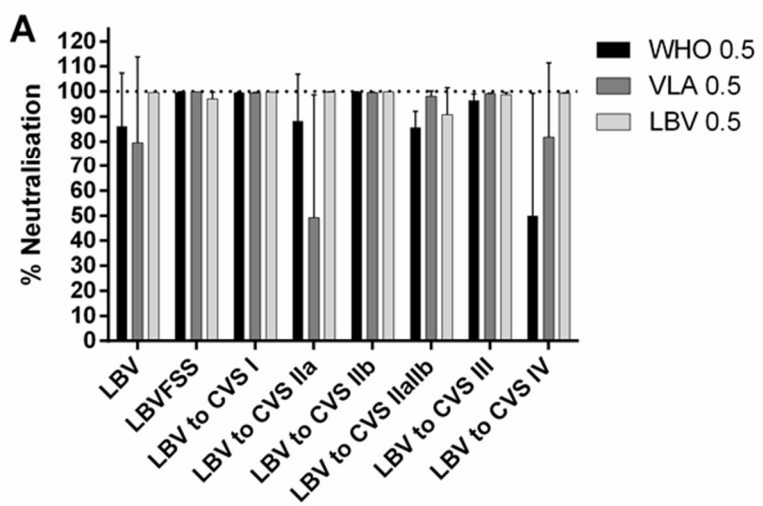


Evans et al Supp. Figure 2



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Evans et al Supp. Figure 3



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